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Microflora of Black and Red Pepper[1]

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Dilution cultures of 30 samples of ground black pepper yielded an average of 39,000 colonies of fungi per g, with a range of 1,700 to 310,000 per g. Total numbers of colonies of bacteria from 11 samples averaged 194,000,000 per g, with a range from 8,300,000 to 704,000,000 per g. A variety of fungi grew from nearly all surface-disinfected whole peppercorns that were cultured. Thirteen samples of ground red pepper from the United States yielded an average of 1,600 colonies of storage fungi per g and an equal number of other fungi; five samples from India yielded an average of 78,900 colonies of storage fungi per g and 169,400 colonies of other fungi per g. Among the fungi from both black and red pepper were *Aspergillus flavus* and *A. ochraceus*, some isolates of which, when grown for 8 to 10 days on moist autoclaved corn and fed to white rats or to 2-day-old Pekin ducklings, were rapidly lethal to them. Aflatoxin B_1 was isolated from one of the samples of corn on which *A. flavus* from black pepper was grown. Among the bacteria isolated from ground black pepper were *Escherichia coli*, *E. freudii*, *Serratia* sp., *Klebsiella* sp., *Bacillus* sp., *Staphylococcus* sp., and *Streptococcus* sp. No cultures of *Shigella* or *Salmonella* were found.

Black pepper is the fruit of *Piper nigrum* L., and is produced chiefly in India and Indonesia; white pepper consists of seeds of the same plant, divested of the tissues that make up the fleshy outer portion of the fresh fruits. Red peppers are the fruit of several species of *Capsicum*, cultivated in many countries. These spices do not undergo any processing, other than drying and grinding, before being added to foods. Over the past 15 years, one of us (C. M. Christensen) has repeatedly cultured various food products to determine the numbers and kinds of fungi present in them. Samples of whole or ground black pepper from various sources usually were included among these, and every sample of black pepper so cultured yielded large numbers of colonies of several species of *Aspergillus*. The *A. glaucus* and *A. restrictus* groups predominated in most samples, but occasionally rather large numbers of colonies of *A. flavus* and *A. ochraceus* were found. In view of the present interest in some of these fungi as possible producers of toxins, a more thorough investigation of the microflora of black and red pepper was thought to be of interest, and the present paper summarizes the results to date.

MATERIALS AND METHODS

Number and source of samples. A total of 55 samples of black pepper were cultured, 50 of ground black pepper and 5 of whole peppercorns. A few samples of whole and ground white pepper were included. Of the 55 samples of black pepper, 30 were bought in stores in or near St. Paul, and comprised 8 brands; 4 came from homes; 3 from passenger planes of different airlines; 1 from a U.S. Navy supply ship; and the rest from restaurants and clubs in Minnesota, Massachusetts, New York, Maryland, and Washington, D.C., in the U.S.; and from London, England; Warsaw, Poland; and New Delhi, India. Nineteen samples of red pepper were cultured; 13 were bought in stores in St. Paul, and were presumably from fruits grown in this country; the other 6 were from India.

Microscopic examination. Whole peppercorns, usually 100 of each sample, were sectioned, and the cavities within the outer rind and within the seed were examined for mycelium, sporophores, or decayed tissues.

Culture media and methods. Several culture media were tested, as described below. Whole peppercorns, usually 100 to 200 of each sample, were cultured with and without previous surface disinfection (surface disinfection consisted of shaking the kernels for 1 min in 2% NaClO, followed by a sterile water rinse). The samples of ground pepper were cultured by various means. If only a small amount of pepper was available, as was true of many of the samples collected in restaurants and planes, or obtained from homes, portions of 10 mg each were weighed out on sterile metal foil and scattered on each of two or more culture dishes. Where larger amounts were available, as with the samples bought in stores, dilution cultures were made as follows: for fungi, 100 mg of ground pepper was suspended in 50 ml of 0.12% solution of sterile agar

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in water contained in milk dilution bottles; the dilute agar solution kept the particles suspended uniformly. Each bottle was shaken briskly 100 times; then two or more portions of 1 ml each were put in sterile petri dishes, melted agar cooled to 50 to 52 C was added, the contents were swirled to suspend the material uniformly, and the agar was allowed to harden. Alternatively, 1-ml portions were pipetted onto the surface of the agar in each of two or more replicate dishes. Some of the lots of pepper, in which the first tests had revealed large numbers of colonies of fungi per gram, were cultured repeatedly, at different dilutions up to 1:5,000, with 4 to 10 plates per dilution. To determine the total number of colonies of bacteria per gram, 1 g of ground pepper was comminuted in 500 ml of the suspension medium in a blender for 1.5 min, and 5 ml of the resulting suspension was placed in 500 ml of suspension medium and shaken 100 times; 5 ml of this was placed in 500 ml of the suspension medium and similarly shaken. Portions of 1 ml of each of the second and third suspensions were pipetted into each of four dishes, and tryptone-glucose-yeast-agar (1) cooled to 50 C was added. The dishes were swirled to distribute the suspension uniformly and were incubated at 30 C. Colonies were counted after 24 and 48 hr. Controls consisted of autoclaved ground pepper or autoclaved sand, cultured by all of the above methods. All dilutions and cultures were made in a sterile air hood. No colonies of fungi and very few colonies of bacteria developed in any of the control cultures.

RESULTS

Microscopic examination. Some kernels of almost every lot of black pepper had relatively conspicuous mycelium or sporophores in cavities of the outer rind, and masses of mycelium were present in at least a few kernels of all of the several samples of whole white pepper examined. One sample of whole white pepper yielded almost no fungi from surface-disinfected seeds, but a mass of mycelium occupied the center of approximately 5% of the split seeds. The central portion of the seeds of a small percentage of both black and white pepper kernels of every sample was discolored and soft, presumably as a result of decay. In one sample of whole black pepper from a local store, an animal dropping, presumably that of a rodent, was found, of about the same size as the peppercorns; in another sample, most of the central portion of one seed had been consumed by an insect, and the resulting cavity was partly filled with insect excreta. Peppercorns presumably hollowed out by insects are shown in Fig. 1.

Protozoa. About 50 kernels of each of the five samples of whole black pepper were put in sterile distilled water in petri dishes, incubated at 25 C, and examined daily. Within 3 days, large ciliated protozoa were numerous in three of the samples, especially in the one from New Delhi, India, that had been harvested only a few months before it was cultured. The protozoa were similar in appearance to those isolated from "weathered" (fungus stained) barley (6), but were not identified.

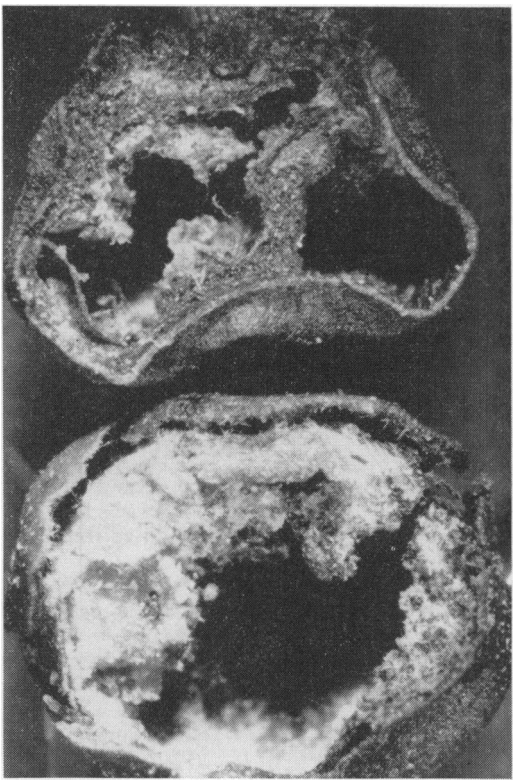

FIG. 1. *Whole black peppercorns, of which only the hulls remain, the interior portion having been consumed, presumably by insects.*

Culture media for fungi. Numbers of colonies of fungi per gram cultured from one sample of ground black pepper on four agar media are given in Table 1. The largest numbers of colonies were obtained on media containing 6% NaCl. Other media tested were acid potato-dextrose-agra (PDA) with 0, 6, and 10% NaCl, and 2% malt extract-agar with 6 and 10% NaCl. Media with high osmotic pressure have long been used to detect osmophilic fungi in stored grains and other materials (3, 4, 8). Some samples of ground pepper yielded larger numbers of colonies of the *A. glaucus* and *A. restrictus* groups when cultured on media with 10% NaCl than on those with 6% NaCl, but many fewer colonies of the *A. flavus* and *A. ochraceus* groups. The medium designated T6A (Difco powdered Tomato Juice Agar, 25 g; agar, 15 g; NaCl, technical grade, 60 g; distilled water, 900 g; plus 30 ppm of chlortetracycline

TABLE 1. *Influence of the medium on the number of colonies of Aspergillus glaucus and A. flavus cultured from one sample of black pepper*

Medium[a]	Colonies per g	
	A. glaucus	*A. flavus*
Cz3	100	500
Cz6A	1,000	250
Cz6	3,000	800
T6A	5,000	650

[a] Cz3 = Czapek's agar with 3% sucrose; Cz6A = Czapek's agar with 3% sucrose, 6% NaCl, and 30 ppm of chlortetracycline; Cz6 = Czapek's agar with 3% sucrose and 6% NaCl; T6A = Difco powdered Tomato Juice Agar, 25 g; Difco agar, 15 g; NaCl, technical grade, 60 g; and distilled water, 900 g; 30 ppm of chlortetracycline was added just before the agar was poured into petri dishes.

TABLE 2. *Fungi isolated from surface-disinfected whole peppercorns*

Sample no.	Percentage of surface-disinfected peppercorns yielding				
	A. glaucus	*A. flavus*	*A. ochraceus*	*A. niger*	*Sporendonema*
1	100	5	31	0	30
2	100	4	11	0	—[a]
3	100	2	5	5	25[b]
4	100	11	13	6	0
8	100	54	84	8	50

[a] *Scopulariopsis* from 10% of surface-disinfected peppercorns.

[b] Plus *Scopulariopsis* from 20% of surace-disinfected peppercorns.

added just before the agar was poured into plates) usually yielded a larger number of colonies of more species of fungi than any of the others tested, and so was used as the standard throughout the subsequent work.

Fungi isolated from whole peppercorns. All whole peppercorns cultured without surface disinfection yielded a heavy growth of fungi, principally *Aspergillus* and *Sporendonema*, from all over their surfaces. The fungi cultured from surface-disinfected peppercorns are listed in Table 2. Cereal grains are not invaded by storage fungi to any significant degree before harvest (10, 11), and the nature of the pepper fruit, with a pulpy flesh and heavy skin, makes it highly probable that it, also, is not invaded by storage fungi before harvest. It seems likely that the samples tested were, subsequent to harvest, exposed to conditions that permitted moderate to heavy invasion by storage and decay fungi. We have encountered *Sporendonema* (in several of our published reports misidentified as *Geotrichum*) in many lots of grains that had undergone deterioration in storage (2), and we consider that its presence in large numbers in such grains is circumstantial evidence that some spoilage has occurred. *Scopulariopsis* (Table 2) is described (9) as ". . . abundans in nature, especially upon vegetation in the later stages of decay. . .," further evidence that some lots of peppercorns may have undergone decay.

Fungi isolated from ground pepper. Numbers of colonies of fungi per gram of black pepper were determined in the 30 samples of which sufficient amounts were available for repeated tests. The average number of colonies per gram in these 30 samples was 39,000 (range, 1,700 to 310,000). Ten of the samples contained more than 10,000 colonies per g and five had more than 100,000 per g. The *A. glaucus* and *A. restrictus* groups predominated in nearly all samples, but relatively large numbers of colonies of *A. flavus* and *A. ochraceus* were obtained from some samples. The sample from which the largest number of colonies of *A. flavus* was cultured (20,000 per g) was a lot of 5 lb bought through the University Food Stores; this same sample was one of the five that yielded more than 100,000 total colonies of fungi per g. Of the other four which yielded such high numbers of colonies per gram, two were 2-oz tins bought in local stores and sealed until opened in the laboratory (both were of the same brand, but were bought in different stores and at different times), one came from the dining room of an armed services officers' club in Washington, D.C., and the other was from a plane of an international airline. *A. niger* was moderately abundant in a few samples, whereas *A. candidus* and *Penicillium* were uncommon.

The numbers of colonies of fungi per gram were also determined in 19 samples of red pepper —13 from the United States and 6 from India. The fungi isolated were classified into two groups: storage fungi (*A. glaucus*, *A. candidus*, *A. flavus*, and *A. ochraceus*) and other fungi (mainly *A. niger*, *Penicillium* spp., and *Rhizopus* spp.). The samples from the United States averaged 1,600 colonies of each type per g. With the samples from India, the average number of colonies per gram was 78,900 for the storage fungi and 169,400 for the other fungi. Two of the samples of red pepper from India yielded slightly more than 200,000 colonies of *A. flavus* per g.

Portions of 10 mg each of a number of samples of ground black and red pepper were scattered directly on the surface of T6A agar in petri dishes, which were then incubated at 25 to 30 C

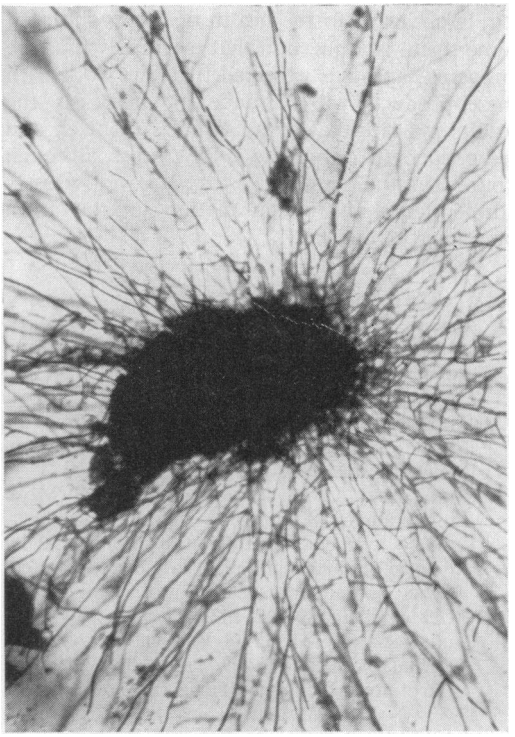

FIG. 2. *Particle of ground black pepper incubated for 20 hr on T6A agar, with a mass of hyphae growing from it.*

for 20 hr and examined microscopically through the bottom of the unopened dishes. From many particles of some samples, masses of hyphae had grown out in that short time, as shown in Fig. 2. Many individual germinating spores were observed also, but these had given rise to only one or two relatively short germ tubes with a few hyphal branches; it seems probable that the masses of hyphae arose either from a relatively large number of spores or from clumps of mycelium. This is circumstantial evidence that the pepper had been invaded by the fungi, not merely contaminated by airborne spores from some other source.

Bacteria. Total numbers of bacteria were determined in 11 samples of black pepper; the average was 194,000,000 per g, with a range from 8,300,000 to 704,000,000 per g (the latter from the sample of 5 lb of black pepper mentioned above). Portions of six samples of black pepper were combined and cultured to determine the presence of various kinds of bacteria; the following were identified: *Escherichia coli, E. freundii, Serratia* sp., *Klebsiella* sp., *Bacillus* sp., *Staphylococcus* sp., and *Streptococcus* sp. Although media and techniques designed for their detection were used, no *Shigella* or *Salmonella* were found.

Toxicity tests. Ten isolates of *A. flavus* were selected from dilution cultures of each of two samples of black pepper bought in local stores; both samples had yielded more than 100,000 colonies of fungi per g, including up to 15,000 colonies of *A. flavus* per gram. Each isolate was inoculated into autoclaved moist corn and incubated for 8 days at 25 C; then the corn was dried and each sample was fed to four 2-day-old Pekin ducklings as the sole ration. Six of the 10 isolates from one sample of pepper, and 1 of 10 from the other resulted in death, in from 1 to 4 days, of all four ducklings to which each was fed. Five isolates of *A. flavus* from one sample of red pepper were similarly grown, and fed to two 21-day-old white rats as their sole ration; three of these resulted in death, in 6 to 7 days, of both of the rats to which each was fed. Ten isolates of *A. ochraceus* from the same sample of red pepper were similarly grown and each was fed to a pair of rats; eight of these resulted in death, in from 4 to 10 days, of both members of the pair to which they were fed, and the remaining two isolates resulted in death of one member of each pair, in 6 to 9 days. In all cases of death, symptoms included subdural hemorrhages, hemorrhage into the gastrointestinal lumen, and hemoglobinuria.

Aflatoxin determination. The corn samples inoculated with *A. flavus* as described above were pooled and analyzed according to the method developed by the Food and Drug Administration (7) for determining the presence of aflatoxin B_1, in an attempt to identify the lethal factor responsible for the death of the Pekin ducklings.

Thin-layer chromatography of the extract revealed the presence of a chemical constituent which had the same R_F value as aflatoxin B_1. The identity was based on comparing the migration distance of the unknown compound with and without an internal standard. The chemical constituents suspected of being aflatoxin B_1 on the chromatograph were eluted off with ethyl alcohol, and the ultraviolet absorption maxima were compared with that of the aflatoxin standard. The absorption characteristics were found to be identical. The extract was also incorporated into clean corn and fed to four Pekin ducklings; death resulted after 3 days. All three tests substantiated the presence of aflatoxin in the corn on which the *A. flavus* isolates had grown.

One isolate of *A. flavus* found to be toxic in the feeding tests above was tested for its ability to produce aflatoxin in the liquid YES medium (5) containing 10% sucrose. Cultures of this isolate were grown for 5 and 7 days at room

temperature and then were extracted with chloroform. The chloroform was concentrated on a flash evaporator, and the constituents of the concentrate were separated by thin-layer chromatography. The extracts of both the 5- and 7-day cultures contained compounds with R_F values identical with that of aflatoxin B_1. These compounds were eluted off the chromatography plates with ethyl alcohol; their ultraviolet absorption spectrum was compared with that of aflatoxin B_1 and was found to be different. The absorption maximum at 360 mμ was lacking, and the 265-mμ band shifted to 270 mμ. Other tests in our laboratory have shown that aflatoxin B_1 can break down when subjected to ultraviolet irradiation or column chromatography on silica gel with absorption changes similar to those found in the culture filtrate above. The data obtained showing the lability of aflatoxin B_1 on chromatography columns suggest that the metabolite obtained from the culture filtrate was a breakdown intermediate of aflatoxin B_1.

The extract of the filtrate from the 5-day-old cultures was incorporated into corn and fed to four Pekin ducklings, all of which died within 2 days. These results support the conclusion that the chemical constituents of the culture filtrate of *A. flavus* having the R_F value identical to that of aflatoxin B_1 was a closely related derivative of the latter.

DISCUSSION

The results here reported constitute a preliminary survey of the microflora of black and red peppers. All of the samples of black pepper, and all of the samples of red pepper that came from India, were heavily invaded by or contaminated with both fungi and bacteria. The fungi comprised mainly species of *Aspergillus*, including *A. flavus* and *A. ochraceus*. Several isolates of both of these group species, from samples in which one or both were abundant, when grown in moist autoclaved corn for 7 to 10 days and fed to ducklings or rats, resulted in rapid death of the test animals. Whether these spices, which may contain up to several hundred thousand fungus spores per gram, including some potentially toxic species, and up to several hundred million bacteria per gram, including *E. coli*, ever constitute a hazard to the health of those who consume them, is a question that eventually must be answered, and it is expected that our work with these products will continue. In any case, the results indicate that many samples of black and red peppers that originate in the tropics, although "pure" in the sense that they are not grossly adulterated with foreign matter, are far from pure microbiologically.

LITERATURE CITED

1. AMERICAN ASSOCIATION OF CEREAL CHEMISTS. 1962. Cereal laboratory methods, 7th ed. American Association of Cereal Chemists, St. Paul, Minn.
2. CHRISTENSEN, C. M., AND H. H. KAUFMANN. 1965. Deterioration of stored grains by fungi. Ann. Rev. Phytopathol. **3**:69–84.
3. CHRISTENSEN, C. M., AND P. LINKO. 1963. Moisture contents of hard red winter wheat as determined by meters and by oven drying, and influence of small differences in moisture content upon subsequent deterioration in storage. Cereal Chem. **40**:129–137.
4. CHRISTENSEN, C. M., G. C. PAPAVIZAS, AND C. R. BENJAMIN. 1959. A new halophilic species of Eurotium. Mycologia **51**:636–640.
5. DAVIS, N. D., U. L. DIENER, AND D. W. ELDRIDGE. 1966. Production of aflatoxins B_1 and G_1 by *Aspergillus flavus* in a semisynthetic medium. Appl. Microbiol. **14**:378–380.
6. KOTHEIMER, J. B., AND C. M. CHRISTENSEN. 1961. Microflora of barley kernels. Wallerstein Lab. Commun. **24**:21–27.
7. NESHEIM, S., D. BANES, L. STOLOFF, AND A. D. CAMPBELL. 1964. Note on aflatoxin analysis in peanuts and peanut products. Assoc. Offic. Analytical Chemists **47**:586.
8. RAPER, K. B., AND D. I. FENNELL. 1965. The genus Aspergillus. The Williams & Wilkins Co., Baltimore.
9. THOM, C. 1930. The penicillia. The Williams & Wilkins Co., Baltimore.
10. TUITE, J. F. 1961. Low incidence of storage molds in freshly harvested seed of soft red winter wheat. Plant Disease Reptr. **43**:470.
11. TUITE, J. F., AND C. M. CHRISTENSEN. 1957. Grain storage studies 23. Time of invasion of wheat seed by various species of Aspergillus responsible for deterioration of stored grain, and source of inoculum of these fungi. Phytopathology **47**:265–268.